WMRC Reports
Waste Management and Research Center

Comparison of Methods for Determination of Anaerobic Herbicide Fate in Flooded Soil

Jennifer J. Crawford
Lutgarde Raskin
F. William Simmons

University of Illinois

RR-90
March 2002
Printed on Recycled Paper

$5

About WMRC's Electronic Publications:

This document was originally published in a traditional format.

It has been transferred to an electronic format to allow faster and broader access to important information and data.

While the Center makes every effort to maintain a level of quality during the transfer from print to digital format, it is possible that minor formatting and typographical inconsistencies will still exist in this document.

Additionally, due to the constraints of the electronic format chosen, page numbering will vary slightly from the original document.

The original, printed version of this document may still be available.

Please contact WMRC for more information:

WMRC
One E. Hazelwood Drive
Champaign, IL 61820
217-333-8940 (phone)

www.wmrc.uiuc.edu

WMRC is a division of the Illinois Department of Natural Resources

RR-90

Comparison of Methods for Determination of Anaerobic Herbicide Fate in Flooded Soil

Jennifer J. Crawford
Lutgarde Raskin

Department of Civil and Environmental Engineering
University of Illinois
Urbana, Illinois

F. William Simmons

Department of Natural Resources and Environmental Sciences
University of Illinois
Urbana, Illinois

March 2002

Submitted to the
Illinois Waste Management and Research Center
One E. Hazelwood Drive
Champaign, IL 61820
www.wmrc.uiuc.edu

Printed by the Authority of the State of Illinois
2002/50

This report is part of WMRC's Research Report Series. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

RNA	ribonucleic acid
EPA	United States Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
DNA	deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
LSC	liquid scintillation counting
TLRC	thin layer radiochromatography
SDS	sodium dodecyl sulfate
OPD	oligonucleotide probe database

ABSTRACT

Microbial populations in anaerobic soil-water microcosms were quantified in order to recommend the best procedure for studying anaerobic herbicide dissipation. Two different methods of establishing anaerobic conditions were used: (i) 30-day pretreatment of the microcosms with 1% glucose before herbicide addition, and (ii) preparation of the microcosms under anaerobic conditions. Nitrate and sulfate were added to half of the anaerobically-prepared microcosms to determine population changes under denitrifying and sulfate-reducing conditions. A portion of these nitrate + sulfate-amended microcosms were sterilized by autoclaving to study the effect of sterilization on the soil populations. Half of each of these four treatments received herbicide, and half did not, so that population changes resulting from herbicide addition could be assessed. Results showed that glucose-pretreated microcosms had a greater percentage of methanogens in the soil at the time of herbicide addition (35%) than the nitrate + sulfate amended microcosms (8%). The pretreatment influences the microbial characteristics of the system by creating a very reducing environment that represents conditions in soils which undergo extended periods of flooding (>3 wks). A typical soil to which herbicide is applied is primarily aerobic and may undergo periods of flooding and anaerobiosis due to heavy rainfall; however, the condition is short-lived (<3 wks) because the soil dries out. Soils that become anaerobic in this manner are not represented by the conditions created by glucose pretreatment. Thus, we recommend that the EPA change the design to include a more naturally-occurring anaerobic environment in fulfilling the anaerobic fate requirement for herbicide registration.

EXECUTIVE SUMMARY

Herbicides may be exposed to anaerobic conditions in flooded soil or in saturated sediments. For this reason, it is important to study herbicide degradation under anaerobic conditions. To achieve this anaerobiosis to investigate anaerobic herbicide fate, the Environmental Protection Agency recommends that soil-water slurries be amended with 1% glucose. The oxygen in the system is rapidly depleted during glucose consumption, and the herbicide of interest is added and monitored after 30 days.

The purpose of our research was to evaluate microbial populations in soil-water microcosms that were prepared to simulate naturally-occurring anaerobic environments and those observed in glucose pretreated microcosms. Soil from the anaerobic soil-water microcosms was extracted to remove ribonucleic acid (RNA). The RNA was applied to nylon membranes which were hybridized with ^{32}P-labeled oligonucleotide probes. Five different probes were used to detect total RNA, methanogens, bacteria, and sulfate reducers *Desulfovibrio* and *Desulfotomaculum*.

Results demonstrated that glucose pretreatment selected for methanogens. Sulfate-reducing bacteria were present in all microcosms, and this population increased over time in sulfate- and herbicide-amended soils. Systems that were subjected to either 30 days of flooding or 30 days flooding with glucose showed decreased numbers of *Desulfovibrio* species. Methanogens proliferate in more reducing environments than are normally found in surface soil and water. We concluded that the conditions created by glucose pretreatment did not represent the majority of herbicide contaminated environments. Since the glucose pretreatment is recommended by the United States Environmental Protection Agency (EPA) in the anaerobic herbicide fate study required for herbicide registration, we

recommend that the EPA revise the design of this study to include anaerobic environments more commonly exposed to herbicides.

BODY OF REPORT

Chapter 1. Introduction

The Midwestern states are subject to soil flooding and periods of soil saturation that influence the fate and transformation of herbicides. Offsite transport of herbicides with sediment in agricultural runoff is a significant cause of nonpoint source pollution. Herbicides and their transformation products may become aquatic pollutants in ditches and surface waters following transport with runoff and subsequent desorption into water bodies. Moreover, soil saturation causes oxygen depletion and development of anoxic conditions which in turn influence herbicide fate in soil.

Overall, there is little information concerning the persistence of herbicides under anaerobic conditions. The registration process for new herbicides includes an evaluation of herbicide stability under anaerobic conditions. The design of the required study usually relies on the preincubation of an aquatic sediment sample with a high concentration of an organic source, such as glucose, to create anaerobic conditions. Since registration studies dictate how herbicides can be used in the environment, these studies should be designed to accurately represent the majority of environments that may be exposed to herbicides. The design of the anaerobic aquatic metabolism study, Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Protocol N-162-3 (USEPA, 1982) creates highly reducing conditions that are uncommon in most natural environments that are exposed to herbicides, such as temporarily flooded soil or surface water sediment. Under anaerobic conditions, many types of microbial metabolisms are possible (Table 1). The anaerobic activities that occur in a given system depend on nutrient and terminal electron acceptor availability and other environmental factors. Anaerobic processes selected for in the anaerobic aquatic metabolism study are currently unknown. Since conditions affect microbial populations, and chemicals may undergo different transformations depending on the active anaerobic populations (Adrian and Suflita, 1994; Berry et al., 1987,

Cozzarelli et al., 1995; Gibson and Suflita, 1986; Häggblom et al., 1993; Kaake et al., 1992), it is important to understand which populations are active in the systems used to evaluate herbicide degradation.

Table 1. Bacterial respiration and utilization of terminal electron acceptors, equilibrium potentials of each half-reaction, and measured redox potentials of these reactions in soil.

Microbial process	**Reduction half-reaction**[a]	**Eh at pH 7 (mV)**	**Measured Eh in soils (mV)**
Aerobic respiration	$1/4O_2 + e^- + H^+ \rightarrow 1/2H_2O$	820	600 - 400
Denitrification	$1/2NO_3^- + e^- + H^+ \rightarrow 1/2NO_2^- + 1/2H_2O$	540	500 - 200
Mn(IV) reduction	$1/2MnO_2 + e^- + 2H^+ \rightarrow 1/2Mn^{2+} + H_2O$	400	400 - 200
Fe(III) reduction	$FeOOH(s) + e^- + 3H^+ \rightarrow Fe^{2+} + 2H_2O$	170	300 - 100
Sulfate reduction	$1/6SO_4^{2-} + 3/2H^+ + e^- \rightarrow 1/6HS^- + 2/3H_2O$	-160	0 - -150
H_2 formation	$H^+ + e^- \rightarrow 1/2H_2$	-410	-150 - -220
Methanogenesis	$1/8CO_2 + H^+ + e^- \rightarrow 1/8CH_4 + 1/4H_2O$	--[b]	-150 - -220

[a] The terminal electron acceptor is the first reactant of each equation.

[b] --, Not calculated.

Use of molecular biology techniques for monitoring microbial abundance

Traditional microbiological identification and enumeration techniques do not allow us to obtain information on microbial concentrations. For example, since traditional culture dependent techniques are by necessity performed in vitro, organisms of interest can only be studied outside of their natural

ecosystem. As a result, important questions remain unanswered because it is by definition impossible to study microbial communities in their entirety outside their ecosystem. To avoid such problems, identification techniques that do not rely on phenotypic characteristics have been developed. They rely on the identification and quantification of molecular signatures unique for specific microbial populations.

Of the techniques currently in use, nucleic acid-based techniques have received the broadest acceptance. They rely on the unique identification of signature sequences present in genes encoded in the deoxyribonucleic acid (DNA) of microorganisms. Oligonucleotide probes are short sequences of DNA that hybridize (i.e., bind) specifically to a target gene product and thus can be used to detect and quantify specific microbial populations. Two particularly useful groups of genes include the genes encoding for ribosomal ribonucleic acid (rRNA) molecules and genes encoding for key metabolic enzymes (e.g., nitrite reductase involved in denitrification). Sequence analysis of rRNA molecules has facilitated the development of a wide assortment of probes targeting phylogenetically (i.e., evolutionarily) related microbial populations (Alm et al., 1996; Amann et al., 1995; DeLong et al., 1989; Raskin et al., 1997). These techniques have considerable potential for use in enumeration of specific microorganisms in environmental samples (Amann et al., 1990, Raskin et al., 1994), and have been used to assess microbial distribution in various environmental applications (de los Reyes et al., 1997, Griffin et al., 1998, Raskin et al., 1995, Teske et al., 1996). Nucleic acid-based techniques are considered state-of-the-art for analysis of complex microbial systems, such as those that exist in soil environments.

Objectives and experimental approach

The purpose of the study is to examine microbial populations in soil-water microcosms and how they respond to anaerobic preparation and incubation over time in the presence and absence of dimethenamid. In this study, two different methods of establishing anaerobic conditions in soil-water microcosms were used: (i) 30-day pretreatment of the microcosms with 1% glucose before herbicide addition, and (ii) preparation of the microcosms under anaerobic conditions. Dissipation of the herbicide ^{14}C-dimethenamid and the associated microbial activity were monitored. The activity of denitrifiers, Fe(III) reducers, sulfate reducers, and methanogens was assessed by functional assays, and population abundance of sulfate reducers, methanogens, and bacteria was determined using oligonucleotide probe hybridizations. The response of microbial communities to herbicide treatment was evaluated by comparing microbial activity in microcosms with and without herbicide.

Results will be used as a basis for recommending a practical, up-to-date protocol to the EPA for industry use in the determination of herbicide fate under anaerobic conditions. The results of this study will provide a more complete understanding of microbial populations in anaerobic environments and how they influence herbicide persistence.

Chapter 2. Methodology

Experimental design

Composite samples were prepared from floodplain and riverbed soil from the Sangamon river. The soil is a Sawmill series sandy clay loam containing a 53% sand, 23% silt, 24% clay, and 5% organic carbon. Three hundred and thirty-two biometers (Fig. 1) were prepared by adding a 1:1 (w/v) ratio of field-moist soil and anaerobic water into 120-ml serum bottles.

Fig. 1. Soil-water biometers employed in this study.

Eighty biometers were prepared with oxygen present (aerobically) and 1% glucose was added to each. This treatment represented the EPA method. The remaining biometers were prepared without oxygen (anaerobically, under N_2; Crawford et al., 2000). Eighty of these were set aside as the unamended treatment. This was the "worst-case" scenario treatment, with no additional amendments, and was included to represent low nutrient environments such as freshwater systems. Eighty-six of the remaining anaerobic biometers were autoclaved 3 times in 4 days (90 min, 121°C). These were considered nonviable and were prepared to determine non-biological degradation of the herbicide. Terminal electron acceptors nitrate and sulfate (to a final concentration of 12 mM each) were added to the autoclaved biometers as well as to the remaining biometers. The viable nitrate + sulfate treatment was considered a "best-case" anaerobic scenario, since the amendment with nitrate and sulfate added the potential for denitrifier and sulfate reducer metabolism in addition to the anaerobic metabolism of Fe(III) reducers, Mn(IV) reducers, and methanogens, the latter of which could occur

in the glucose pretreated and unamended treatments. Moreover, the nitrate and sulfate amendments represented flooded surface soil environments in which nitrate and sulfate may be available. Half of all biometers for the 4 treatments received the herbicide ^{14}C-dimethenamid in a filter-sterile (0.2 µm, PVDF) solution of methanol, and the remainder were not amended with herbicide in order to compare microbial activity in the presence and absence of dimethenamid.

Sampling and analyses

For each of the treatments, microcosms were sacrificed in triplicate and processed at time 0, 1, 2, 4, 8, 16, 24, 32, 64, 96, 142, 188, and 365 days. Methane (both [^{14}C] and unlabeled) was quantitatively determined at 142 DAT with a gas chromatography combustion technique (Freedman and Gossett, 1989). Dimethenamid volatilization and estimation of the radioactivity associated with hydrophobic ^{14}C volatiles were assessed with polyurethane foam plugs (PUFs) and the method of Mervosh et al. (1995). To obtain these samples, the headspace of each microcosm was displaced with N_2 gas into a 20-ml syringe that contained a PUF plug. The PUF was transferred to a scintillation vial for liquid scintillation counting (LSC).

Upon opening each microcosm, the redox potential was immediately measured with a polished combination platinum AgCl/Ag redox electrode connected to a portable Orion pH/ISE meter (model 290A). The solution in each CO_2 collection vial was quantitatively transferred to a scintillation vial, and $^{14}CO_2$ was quantified via LSC.

^{14}C-dimethenamid and metabolite distribution were quantified in the headspace, aqueous, sorbed, and bound (unextractable) fractions using LSC after processing as follows. After redox measurement, the solid and liquid phases of the soil slurry were separated by centrifugation (15 min, 4000 x *g*) and pH

was measured in the aqueous fraction. Aqueous samples were filtered (0.2 μm), and portions were stored at 4°C for herbicide analysis by thin-layer radiochromatography (TLRC), acidified and frozen for volatile fatty acid analysis, or stored frozen until terminal electron accepting process analysis (below). Aqueous aliquots for liquid scintillation counting were prepared in duplicate. One was treated with saturated $BaCl_2$ to precipitate CO_2/HCO_3^-, centrifuged (4 min, 12,000 x *g*), and the supernatant was drawn off for LSC. The difference in ^{14}C between the $BaCl_2$-treated and untreated samples was reported as $H^{14}CO_3^-$ (or aqueous $^{14}CO_2$).

Aqueous samples were extracted 1:6 (v/v) with acetone-fortified ethyl acetate (5%, v/v), and the organic portion was concentrated by evaporating to dryness and dissolving in 100 μl of ethyl acetate for TLRC. Soil was extracted with ethyl acetate/acetone for 48 hours with horizontal shaking. After centrifuging (15 min, 4000 x *g*), a 15-ml portion of the ethyl acetate was transferred to a glass vial. An aliquot was removed for LSC, and the remainder was evaporated to dryness and dissolved in 2 ml of ethyl acetate/5% acetone for TLRC. After the soil was air dry and pulverized (with mortar and pestle), bound (unextractable) ^{14}C-residues were quantified by combustion (Harvey Biological Oxidizer OX500, R. J. Harvey Instruments, Hillsdale, NJ) and LSC of collected $^{14}CO_2$.

Analysis of herbicide and metabolites

The ratio of ^{14}C-dimethenamid to ^{14}C-metabolites was quantified in aqueous and organic extracts using TLRC (Crawford et al., submitted). A mobile phase composition of ethyl acetate:toluene:formic acid (conc.):H_2O of 87:3:5:5 (Sandoz Agro., personal communication) was employed with Adsorbosil Plus 1 P (Alltech Associates, Inc., Deerfield, IL) TLC plates.

Determination of terminal electron accepting processes

Methanogenesis was determined as above. Terminal electron acceptors NO_3^- and SO_4^{2-} as well as Fe^{2+} formation were monitored in aqueous samples with microscale methods (described in detail in Crawford et al., submitted and Loor-Vela et al., submitted). Spectrophotometric measurements of microtiter plate assays were obtained with a CERES Model UV900HDi plate reader (Bio-Tek Instruments, Winooski, VT). Nitrate-nitrogen determination was accomplished with the modified method of Vendrall and Patrick (1990). Sulfate was determined photometrically with a microscale adaption of the barium precipitation procedure described by Cypionka and Pfennig (1986). Extractable soil Fe^{2+} was analyzed with a microtiter-plate modification of Lovley and Phillips (1987).

Sampling and RNA extraction from sediment

During sampling, 0.5-g aliquots of each soil sample were distributed into screw-cap centrifuge tubes and stored at -80C. Cell lysis was facilitated with bead beating and 20% sodium dodecyl sulfate detergent (SDS). RNA was extracted with a low-pH hot-phenol extraction method based on the procedure of Ogram et al. (1995), using guanidine thiocyanate-β-mercaptoethanol-Sarkosyl-phenol-chloroform RNAse inhibitor (Alm and Stahl, unpublished). After RNA precipitation, the pellet was resuspended in RNAse-free water (Sigma Chemical). Sephracryl S-300 DNA spun columns (Pharmacia Biotech) were pretreated according to manufacturer instructions and were used to remove humic material from the RNA extracts. Following purification with the spun column, DNAse I (Ambion) was added to each extract and the solution was incubated at 37°C for 15 min to remove DNA. RNA was again precipitated from the solution and resuspended in RNAse-free water. Quantification was achieved with a Ribo-Green quantification kit (Molecular Probes, Inc.).

Hybridizations with oligonucleotide probes representing anaerobic microorganisms common to

soil and sediment

Quantitative membrane hybridizations were conducted as previously described (Raskin et al., 1997). RNA was denatured, immobilized on nylon membranes, and hybridized with oligonucleotide probes (Table 2) radiolabeled with ^{32}P . Hybridization signals were quantified using an InstantImager Electronic Autoradiography System (Packard Instrument Company). Detailed information on the probes and original references are available through the Oligonucleotide Probe Database (OPD) (Alm et al., 1996).

Table 2. Olignucleotide probes used for RNA analysis.

PROBE	TARGET GROUP	WASH TEMPERATURE (°C)
S-*-Univ-1390-a-A-18	Virtually all organisms	44
S-*-Dsv-0698-a-A-20	Most *Desulfovibrio* spp.	55
S-G-Dtm-0229-a-A-18	*Desulfotomaculum* spp.	53
S-D-Arch-0915-a-A-20	*Archaea*	58
S-D-Bact-0338-a-A-18	*Bacteria*	55

Data treatment

The statistical program SAS®/STAT® for Windows (SAS, 1988) was used to generate means, standard errors, linear regressions, and % of total radiocarbon applied that was recovered as mineralized, sorbed (parent and metabolite), aqueous parent, and bound residue ^{14}C at each sample date. Hybridized RNA was quantified with external standards. The percent of target RNA was calculated with the following equation:

$$\% \text{ Target} = \frac{\text{RNA detected with the specific probe}}{\text{RNA detected with the universal probe}} \times 100\%$$

Chapter 3. Results and Discussion

RNA quantity

In this study, we used nucleic acid-based probes to quantify the abundance of both general and specific populations of soil microorganisms (Table 2) in glucose pretreated microcosms +/- herbicide and in nitrate + sulfate-amended microcosms +/- herbicide. The Universal probe is routinely used to detect all organisms (not just microorganisms). Virtually all microbial RNA can be detected using the *Bacteria* and *Archaea* probes together. Methanogenic organisms are detected by the *Archaea* probe. We also chose to quantify 2 specific populations of bacteria that are able to reduce sulfate, *Desulfovibrio* spp., which are Gram-, and *Desulfotomaculum* spp., which are Gram+.

Oligonucleotide probe hybridizations involve applying each sample of RNA in triplicate to several membranes which are each hybridized with a specific ^{32}P-labeled oligonucleotide probe. Standards are applied to each membrane and the quantity of sample RNA is determined from a standard curve. Quantities are expressed as the % of target RNA (Figs. 2-9), and this means the % of the RNA obtained with a specific probe relative to the total RNA obtained for the sample. This number is obtained by the dividing the quantity obtained by the specific probe by the quantity obtained with the universal probe and multiplying by 100. The amount of RNA necessary for hybridization with the 5 probes we used (4 specific probes + the universal probe) was at least 200 ng/sample and was obtained in only 102 of 200 extractions. The treatments we analyzed represented 4 of the original 8 treatments. These 4 contained more RNA than the other 4 treatments, perhaps due to the amendments of glucose or terminal electron acceptors nitrate and sulfate which are usually limiting in soil. The treatments in which sufficient amounts of rRNA were not recovered were the unamended +/- herbicide and autoclaved +/- herbicide. Since we could fulfill the objectives of our project without these treatments, we concentrated on the two treatments in which sufficient rRNA was available, the

glucose-pretreated and nitrate + sulfate-amended. In the future we plan to analyze the other treatments after improving the efficiency of our extraction protocol.

Initial microcosm conditions and microbial populations

Microbial populations were quantified in soil-water microcosms without any pretreatment, after a 30-day glucose pretreatment, and after a 30-day flooding period (no glucose). A significant increase in the relative abundance of methanogens was observed in the glucose pretreated microcosms compared with the 30 days flooding, no glucose, and the soil without flooding or glucose treatment (Fig. 2). There was no significant difference in methanogen populations due to the 30-day flooding period without glucose. Hybridizations with the *Bacteria* probe demonstrated significant differences between treatments (Fig. 3). Two specific genus of sulfate-reducing bacteria are expected in soil and were present in all microcosms (Figs. 4 and 5). *Desulfovibrio* spp. were more abundant in the soil-water microcosms before flooding and flooding with glucose pretreatment (Fig. 4). Glucose pretreatment resulted in substantially less recovery of this genus of sulfate-reducers. In contrast, flooding and flooding with glucose pretreatment did not appear to affect *Desulfotomaculum* populations in the soil (Fig. 5). *Desulfotomaculum* spp. are spore formers and are better equipped to sustain changes in environmental conditions than non-sporeformers.

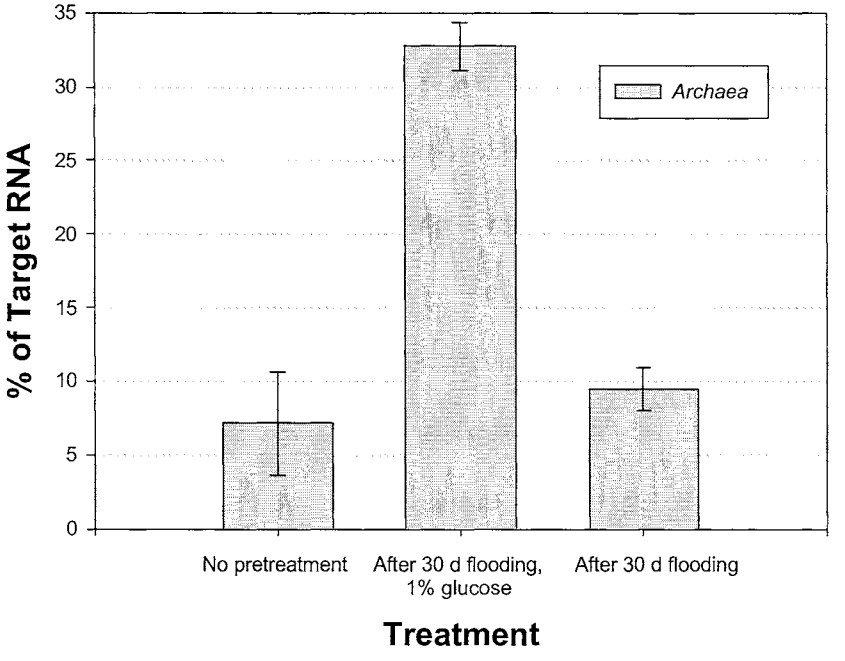

Fig. 2. Abundance of *Archaea* in soil-water microcosms as influenced by soil flooding and glucose pretreatment. Error bars represent the standard deviation of the mean.

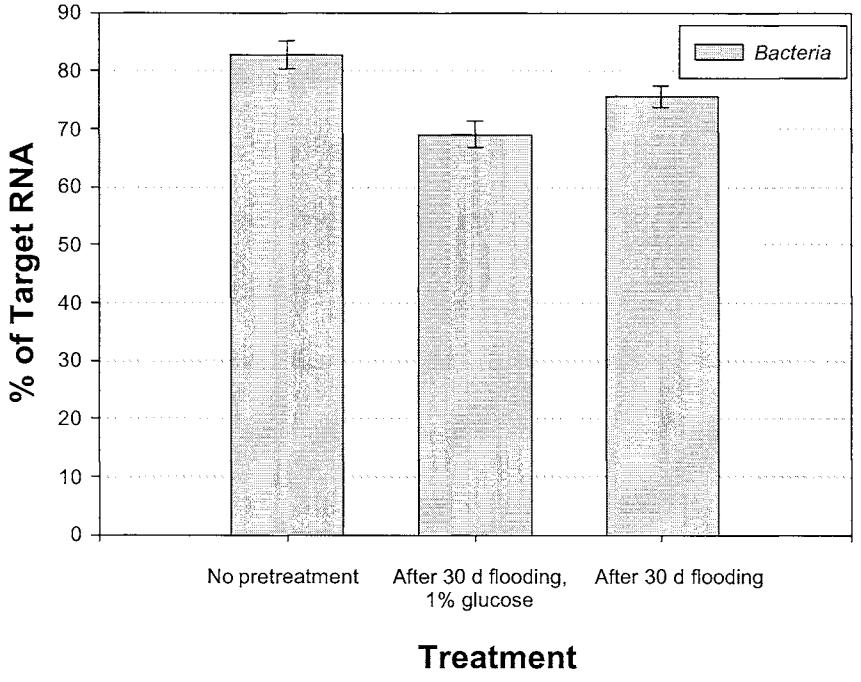

Fig. 3. Abundance of *Bacteria* in soil-water microcosms as influenced by soil flooding and glucose pretreatment. Error bars represent the standard deviation of the mean.

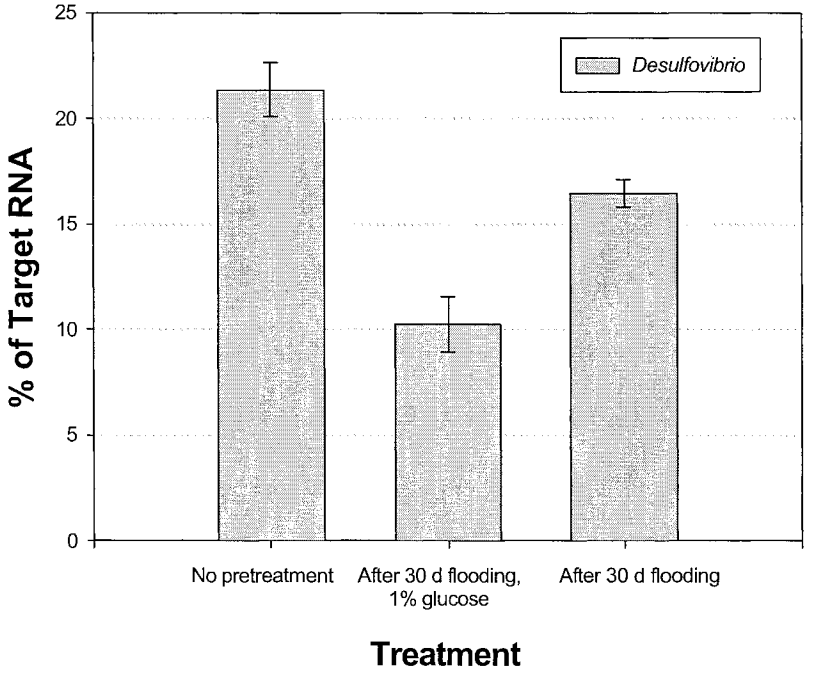

Fig. 4. Abundance of *Desulfovibrio* spp. in soil-water microcosms as influenced by soil flooding and glucose pretreatment. Error bars represent the standard deviation of the mean.

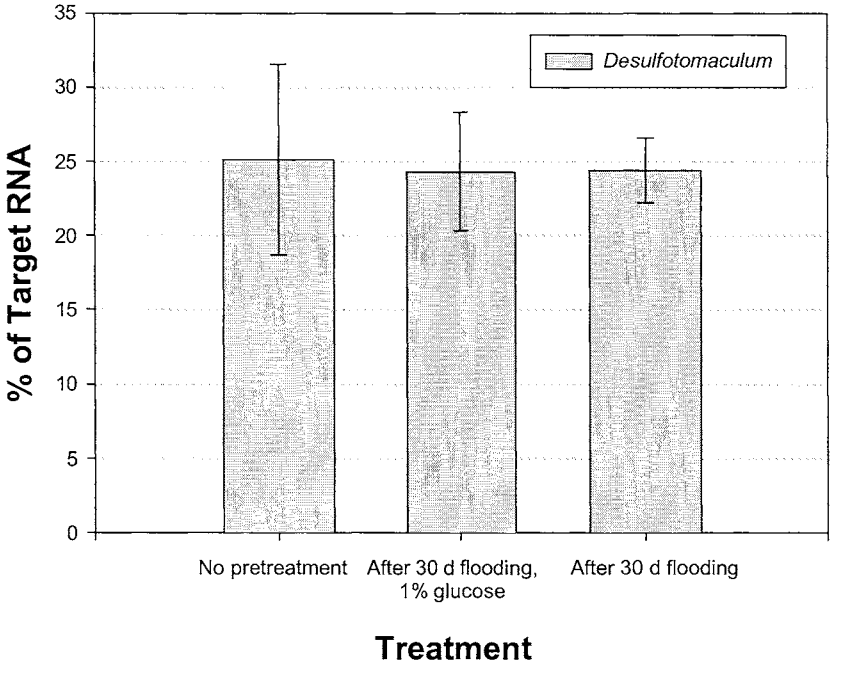

Fig. 5. Abundance of *Desulfotomaculum* spp. in soil-water microcosms as influenced by soil flooding and glucose pretreatment. Error bars represent the standard deviation of the mean.

Degradation of [^{14}C]dimethenamid

Depletion of dimethenamid occurred at similar rates in viable microcosms, and was slower in autoclaved microcosms (Fig. 6a). Transformations of dimethenamid were evident with metabolite formation over time, except in the autoclaved microcosms, in which metabolite levels were negligible (Fig. 6b). Up to 7 metabolites were observed in each treatment, with 2 major metabolites that accumulated to greater than 5 % of total ^{14}C (data not shown) in viable treatments. The levels of different metabolites were slightly different between treatments. This difference in metabolites was observed previously in our laboratory (Crawford et al., submitted); however, the difference was not significant enough to warrant a change in the EPA protocol. Therefore, the study was repeated with the purpose of quantifying microbial populations, which is the focus of the present study. For this reason, microbial community dynamics in the different microcosms and not degradation of dimethenamid are detailed in this report. Degradation of dimethenamid was monitored to confirm that similar trends occurred in both studies. Mineralization of ^{14}C as measured by $^{14}CO_2$ evolution was inconsequential to dimethenamid degradation (less than 1 % of ^{14}C). The remaining balance of ^{14}C (up to 60 %) accumulated as ^{14}C-bound (unextractable) residue.

Terminal electron accepting processes over time

Depletion of nitrate and sulfate as well as formation of nitrite, Fe^{2+}, and methane were monitored in all microcosms (Tables 3-6). The succession of redox conditions and corresponding anaerobic processes occurred as expected in anaerobic soils, in the order of denitrification, Fe(III) reduction (as determined by Fe^{2+} formation), sulfate reduction, and methanogenesis. Denitrification and sulfate reduction were only observed in the nitrate + sulfate-amended treatment, though the other treatments contained background levels of sulfate. Methanogenesis was quantified starting with day

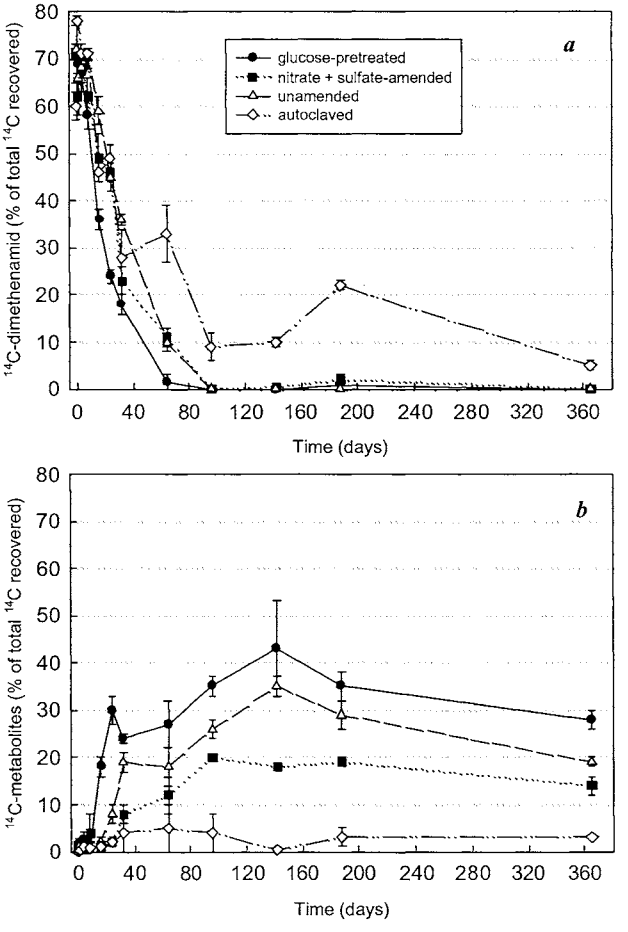

Fig. 6 a,b. Depletion of [^{14}C]dimethenamid (a) and formation of [^{14}C]metabolites (b) over time in soil-water microcosms. Day 0 indicates the start of the experiment by herbicide addition (where appropriate) and occurs on day 30 for glucose-pretreated microcosms. Each data point is the mean of triplicate samples, and error bars represent the standard error of the mean. Metabolite data represents the sum of up to 7 metabolites in each treatment.

Table 3. Concentrations of terminal electron acceptors and reduced compounds in the glucose-pretreated test system without and with [^{14}C]dimethenamid.

Time	without [^{14}C]dimethenamid			with [^{14}C]dimethenamid		
(days)	Fe(II) mmol kg^{-1}	Sulfate mM	% [CH_4][a]	Fe(II) mmol kg^{-1}	Sulfate mM	% [CH_4][a]
0	1.8 ± 0.6	1.1 ± 0.04	nd	2.2 ± 0.4	1.7 ± 0.4	nd
4	1.3 ± 0.1	0.6 ± 0.09	nd	5.9 ± 2.8	0.4 ± 0.05	nd
8	10.4 ± 1.2	0.6 ± 0.04	nd	8.8 ± 1.6	0.4 ± 0.1	nd
16	5.1 ± 0.1	0.4 ± 0.06	nd	4.8 ± 0.1	0.2 ± 0.004	nd
24	nd[b]	0.4 ± 0.05	nd	nd	0.2 ± 0.002	nd
32	9.9 ± 1.3	0.8 ± 0.2	nd	8.6 ± 0.3	0.4 ± 0.04	nd
64	9.5 ± 6.5	1.7 ± 0.3	nd	25.1 ± 0.4	0.2 ± 0.01	nd
96	36.6 ± 1.1	1.4 ± 0.7	4.3 ± 2.2	33.7 ± 1.5	0.4 ± 0.01	2.9 ± 2.9
142	nd	0.5 ± 0.1	nd	nd	0.4 ± 0.01	nd
188	10.5 ± 1.01	0.7 0.001	41.2 2.8	9.3 0.5	0.3 0.01	9.2 9.2
365	37.9 ± 12.1	1.6 ± 0.2	16.9 ± 1.7	30.3 ± 1.0	0.5 ± 0.04	10.8 ± 1.9

[a] concentration was measured in biometer headspace. [b] not determined.

Table 4. Concentrations of terminal electron acceptors and reduced compounds in the unamended test system without and with [^{14}C]dimethenamid.

Time	without [^{14}C]dimethenamid			with [^{14}C] dimethenamid		
(days)	Fe(II) mmol kg^{-1}	Sulfate mM	% [CH_4][a]	Fe(II) mmol kg^{-1}	Sulfate mM	% [CH_4][a]
0	1.5 ± 0.1	1.5 ± 0.1	nd	1.5 ± 0.1	1.3 ± 0.2	nd
4	2.2 ± 0.1	1.1 ± 0.3	nd	3.3 ± 0.2	1.3 ± 0.2	nd
8	1.8 ± 0.5	1.7 ± 0.4	nd	2.3 ± 0.5	1.1 ± 0.04	nd
16	0.4 ± 0.2	1.2 ± 0.2	nd	3.1 ± 0.5	0.3 ± 0.03	nd
24	1.4 ± 0.1	0.6 ± 0.3	nd	10.2 ± 5.1	2.4 ± 2.3	nd
32	3.1 ± 0.1	0.2 ± 0.02	nd	5.4 ± 0.6	0.1 ± 0.01	nd
64	21.4 ± 1.0	0.3 ± 0.01	2.0 ± 0.0	22.8 ± 1.3	4.9 ± 2.4	6.7 ± 3.8
96	39.7 ± 5.6	0.3 ± 0.1	3.1 ± 2.2	37.8 ± 3.3	0.3 ± 0.02	0.0 ± 0.0
142	46.4 ± 2.2	0.2 ± 0.01	nd	46.5 ± 2.0	0.2 ± 0.1	nd
188	9.1 1.5	0.4 0.03	6.8 ± 0.4	8.5 1.8	0.5 0.2	0.0 ± 0.0
365	32.6 ± 2.6	0.5 ± 0.02	5.3 ± 0.3	13.2 ± 4.1	3.5 ± 0.4	0.0 ± 0.0

[a] concentration was measured in biometer headspace. [b] not determined.

Table 5. Concentrations of terminal electron acceptors and reduced compounds in the nitrate + sulfate-amended test system with and without [^{14}C]dimethenamid.

Time	without [^{14}C]dimethenamid				with [^{14}C]dimethenamid			
(days)	Nitrate mM	Fe(II) mmol kg^{-1}	Sulfate mM	% [CH_4][a]	Nitrate mM	Fe(II) mmol kg^{-1}	Sulfate mM	% [CH_4][a]
0	10.7 ± 3.4	1.5 ± 0.1	7.0 ± 0.9	nd	10.7 ± 3.4	1.5 ± 0.1	9.6 ± 0.9	nd
4	4.6 ± 4.6	0.7 ± 0.5	9.8 ± 1.4	nd	0.5 ± 0.3	3.4 ± 0.5	9.9 ± 0.6	nd
8	18.2 ± 1.0	0.3 ± 0.1	10.8 ± 0.9	nd	0.1 ± 0.0	2.2 ± 0.1	12.0 ± 1.6	nd
16	2.2 ± 1.6	0.0 ± 0.0	12.7 ± 1.7	nd	0.1 ± 0.0	4.5 ± 0.3	7.0 ± 1.3	nd
24	2.3 ± 2.3	2.6 ± 0.3	6.6 ± 0.6	nd	0.1 ± 0.0	5.0 ± 0.7	12.1 ± 2.9	nd
32	0.8 ± 0.6	0.3 ± 0.2	6.2 ± 0.5	nd	0.0 ± 0.0	26.6 ± 6.7	3.2 ± 1.7	nd
64	0.0 ± 0.0	7.9 ± 0.4	7.3 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	25.5 ± 3.9	0.1 ± 0.0	5.3 ± 2.1
96	0.0 ± 0.0	35.2 ± 0.4	9.4 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	41.7 ± 4.6	2.2 ± 1.7	1.0 ± 0.5
142	0.0 ± 0.0	41.5 ± 1.0	5.0 ± 2.5	nd	0.0 ± 0.0	7.7 ± 0.9	1.0 ± 0.4	nd
188	0.0 ± 0.0	10.8 ± 0.5	4.2 ± 0.8	2.4 ± 2.4	0.0 ± 0.0	13.4 ± 0.5	0.5 ± 0.1	13.9 ± 5.0
365	0.0 ± 0.0	32.3 ± 3.4	0.5 ± 0.1	2.6 ± 1.6	0.0 ± 0.0	22.9 ± 4.7	0.7 ± 0.1	11.8 ± 4.0

[a] concentration was measured in biometer headspace. [b] not determined.

Table 6. Concentrations of terminal electron acceptors and reduced compounds in the autoclaved test system with and without [^{14}C]dimethenamid.

Time (days)	without [^{14}C]dimethenamid				with [^{14}C]dimethenamid			
	Nitrate mM	Fe(II) mmol kg^{-1}	Sulfate mM	% [CH_4][a]	Nitrate mM	Fe(II) mmol kg^{-1}	Sulfate mM	% [CH_4][a]
0	2.2 ± 0.4	nd[b]	8.9 ± 3.5	nd	7.0 ± 2.2	nd	10.0 ± 3.2	nd
4	28.0 ± 2.1	2.5 ± 0.4	14.7 ± 0.7	nd	0.4 ± 0.0	1.1 ± 0.4	8.6 ± 1.5	nd
8	21.9 ± 1.1	1.6 ± 0.1	27.2 ± 1.8	nd	8.1 ± 7.0	1.5 ± 0.2	23.5 ± 2.7	nd
16	adl[c]	0.6 ± 0.1	15.1 ± 5.1	nd	0.1 ± 0.0	0.5 ± 0.2	13.8 ± 4.5	nd
24	adl	5.3 ± 1.2	12.0 ± 0.8	nd	0.2 ± 0.0	6.3 ± 1.9	0.1 ± 0.0	nd
32	0.3 ± 0.1	7.8 ± 0.7	7.0 ± 3.3	nd	0.1 ± 0.0	15.6 ± 0.7	9.8 ± 0.3	nd
64	0.5 ± 0.3	1.4 ± 0.2	13.4 ± 2.9	0.0 ± 0.0	0.2 ± 0.0	1.2 ± 0.7	10.9 ± 1.4	0.0 ± 0.0
96	adl	8.4 ± 0.5	22.4 ± 1.5	0.0 ± 0.0	0.4 ± 0.2	22.0 ± 2.0	16.8 ± 3.4	0.0 ± 0.0
142	1.0 ± 0.3	3.0 ± 0.4	15.8 ± 3.2	nd	0.1 ± 0.0	3.9 ± 1.0	19.2 ± 4.0	nd
188	0.4 ± 0.1	3.1 ± 0.9	10.9 ± 0.6	0.0 ± 0.0	0.1 ± 0.0	5.5 ± 0.8	0.9 ± 0.1	0.0 ± 0
365	0.0 ± 0.0	2.3 ± 0.1	30.9 ± 1.7	0.0 ± 0.0	2.2 ± 0.2	1.4 ± 0.2	12.3 ± 2.2	0.0 ± 0

[a] concentration was measured in biometer headspace. [b] not determined. [c] above detection limit (28 mM).

64 or 96 (due to the lack of GC availability until that time). However, in a study conducted since the one described here, elevated methane levels were observed following a 30 day glucose preincubation at the time of herbicide addition (Loor-Vela et al., submitted). Methane accumulated in all viable treatments except the unamended dimethenamid-treated microcosms (Tables 3-5). Depletion of nitrate and sulfate occurred significantly faster in the herbicide-treated microcosms (Table 5), but Fe^{2+} formation did not appear to be influenced by dimethenamid treatment (Tables 3-5). The effect of dimethenamid treatment on methanogenesis was unclear since methane accumulated to higher levels in the dimethenamid-free glucose-pretreated and unamended microcosms but in the dimethenamid-treated nitrate + sulfate amended microcosms. In the autoclaved microcosms, nitrate was depleted, but Fe^{2+} formation was sporadic and sulfate reduction and methanogenesis were not evident over time compared with the other treatments (Table 6). The lack of metabolite formation in these microcosms and the significantly less rRNA recovered and evidence of metabolic activity indicate that these microcosms were predominantly nonviable. Autoclaving causes chemical changes to soil that may have contributed to nitrate transformation.

Volatile fatty acids in soil-water microcosms over time

Formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid were monitored in all microcosms during the 365 d incubation (Tables 7-14). All but valeric acid were observed in the glucose-pretreated microcosms (Tables 7 and 8). Formic, acetic, propionic, isoburtyric, butyric, and isovaleric acids accumulated initially and were depleted over time. Higher concentrations of volatile fatty acids (VFAs) were observed in the dimethenamid-treated, glucose-pretreated microcosms (Tables 7 and 8). In the unamended treatments,

Table 7. Concentrations of volatile fatty acids in the glucose-pretreated test system without [^{14}C]dimethenamid.

Time (days)	Formic acid mg L^{-1}	Acetic acid mg L^{-1}	Propionic acid mg L^{-1}	Isobutyric acid mg L^{-1}	Butyric acid mg L^{-1}	Isovaleric acid mg L^{-1}	Valeric acid mg L^{-1}
0	0 ± 0	57 ± 15	187 ± 22	20 ± 10	290 ± 146	0 ± 0	0 ± 0
1	20 ± 10	133 ± 55	220 ± 15	53 ± 19	293 ± 147	10 ± 10	0 ± 0
4	0 ± 0	60 ± 46	80 ± 80	20 ± 20	0 ± 0	0 ± 0	0 ± 0
8	0 ± 0	0 ± 0	80 ± 40	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	0 ± 0	0 ± 0	13 ± 13	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	0 ± 0	3 ± 3	0 ± 0	0 ± 0	33 ± 33	0 ± 0	0 ± 0
64	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
96	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
142	0 ± 0	0 ± 0	0 ± 0	100 ± 100	0 ± 0	0 ± 0	0 ± 0
188	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
365	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 8. Concentrations of volatile fatty acids in the glucose-pretreated test system with [^{4}C]dimethenamid.

Time (days)	Formic acid mg L^{-1}	Acetic acid mg L^{-1}	Propionic acid mg L^{-1}	Isobutyric acid mg L^{-1}	Butyric acid mg L^{-1}	Isovaleric acid mg L^{-1}	Valeric acid mg L^{-1}
0	20 ± 20	67 ± 42	220 ± 12	47 ± 3	597 ± 26	0 ± 0	0 ± 0
1	10 ± 10	250 ± 32	223 ± 12	43 ± 3	230 ± 14	13 ± 9	0 ± 0
4	0 ± 0	253 ± 123	263 ± 20	40 ± 20	87 ± 49	33 ± 17	0 ± 0
8	0 ± 0	283 ± 55	360 ± 68	27 ± 27	0 ± 0	0 ± 0	0 ± 0
16	0 ± 0	87 ± 45	427 ± 15	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	0 ± 0	27 ± 7	10 ± 10	0 ± 0	0 ± 0	0 ± 0	0 ± 0
64	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
96	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
142	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
188	0 ± 0	0 ± 0	33 ± 33	83 ± 83	0 ± 0	0 ± 0	0 ± 0
365	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 9. Concentrations of volatile fatty acids in the unamended test system without [^{14}C]dimethenamid.

Time (days)	Formic acid mg L^{-1}	Acetic acid mg L^{-1}	Propionic acid mg L^{-1}	Isobutyric acid mg L^{-1}	Butyric acid mg L^{-1}	Isovaleric acid mg L^{-1}	Valeric acid mg L^{-1}
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	17 ± 17	0 ± 0
4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	0 ± 0	47 ± 23	0 ± 0	27 ± 27	67 ± 67	0 ± 0	0 ± 0
16	0 ± 0	0 ± 0	0 ± 0	0 ± 0	23 ± 23	0 ± 0	0 ± 0
24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	0 ± 0	7 ± 3	0 ± 0	0 ± 0	150 ± 93	0 ± 0	0 ± 0
64	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
96	0 ± 0	0 ± 0	0 ± 0	0 ± 0	667 ± 66	0 ± 0	0 ± 0
142	0 ± 0	0 ± 0	0 ± 0	103 ± 103	117 ± 11	0 ± 0	0 ± 0
188	0 ± 0	0 ± 0	0 ± 0	103 ± 52	0 ± 0	0 ± 0	0 ± 0
365	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 10. Concentrations of volatile fatty acids in the unamended test system with [^{14}C]dimethenamid.

Time (days)	Formic acid mg L^{-1}	Acetic acid mg L^{-1}	Propionic acid mg L^{-1}	Isobutyric acid mg L^{-1}	Butyric acid mg L^{-1}	Isovaleric acid mg L^{-1}	Valeric acid mg L^{-1}
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	0 ± 0	210 ± 36	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24	0 ± 0	260 ± 35	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	0 ± 0	10 ± 6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
64	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
96	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
142	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
188	0 ± 0	0 ± 0	0 ± 0	113 ± 64	0 ± 0	0 ± 0	0 ± 0
365	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 11. Concentrations of volatile fatty acids in the nitrate + sulfate-amended test system without [^{14}C]dimethenamid.

Time (days)	Formic acid mg L^{-1}	Acetic acid mg L^{-1}	Propionic acid mg L^{-1}	Isobutyric acid mg L^{-1}	Butyric acid mg L^{-1}	Isovaleric acid mg L^{-1}	Valeric acid mg L^{-1}
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	7 ± 7	0 ± 0	0 ± 0
1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
64	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
96	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
142	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
188	0 ± 0	0 ± 0	13 ± 13	0 ± 0	0 ± 0	0 ± 0	0 ± 0
365	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 12. Concentrations of volatile fatty acids in the nitrate + sulfate-amended test system with [^{14}C]dimethenamid.

Time (days)	Formic acid mg L^{-1}	Acetic acid mg L^{-1}	Propionic acid mg L^{-1}	Isobutyric acid mg L^{-1}	Butyric acid mg L^{-1}	Isovaleric acid mg L^{-1}	Valeric acid mg L^{-1}
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24	0 ± 0	30 ± 15	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
64	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
96	0 ± 0	727 ± 639	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
142	0 ± 0	543 ± 272	27 ± 7	0 ± 0	0 ± 0	0 ± 0	0 ± 0
188	17 ± 12	0 ± 0	10 ± 10	0 ± 0	0 ± 0	0 ± 0	37 ± 37
365	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 13. Concentrations of volatile fatty acids in the autoclaved test system without [^{14}C]dimethenamid.

Time (days)	Formic acid mg L^{-1}	Acetic acid mg L^{-1}	Propionic acid mg L^{-1}	Isobutyric acid mg L^{-1}	Butyric acid mg L^{-1}	Isovaleric acid mg L^{-1}	Valeric acid mg L^{-1}
0	20 ± 12	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	113 ± 7
1	7 ± 7	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	120 ± 0
4	17 ± 17	50 ± 29	0 ± 0	0 ± 0	27 ± 15	0 ± 0	110 ± 10
8	60 ± 0	77 ± 3	0 ± 0	0 ± 0	43 ± 23	0 ± 0	110 ± 0
16	63 ± 19	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
24	30 ± 17	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
32	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	43 ± 3
64	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	37 ± 3
96	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	367 ± 23
142	0 ± 0	0 ± 0	0 ± 0	50 ± 50	0 ± 0	0 ± 0	143 ± 34
188	67 ± 33	0 ± 0	0 ± 0	0 ± 0	77 ± 77	0 ± 0	117 ± 17
365	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 14. Concentrations of volatile fatty acids in the autoclaved test system with [^{14}C]dimethenamid.

Time (days)	Formic acid mg L^{-1}	Acetic acid mg L^{-1}	Propionic acid mg L^{-1}	Isobutyric acid mg L^{-1}	Butyric acid mg L^{-1}	Isovaleric acid mg L^{-1}	Valeric acid mg L^{-1}
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	120 ± 0
1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
4	3 ± 3	63 ± 9	0 ± 0	80 ± 6	147 ± 47	0 ± 0	123 ± 62
8	13 ± 13	77 ± 38	0 ± 0	27 ± 27	130 ± 81	0 ± 0	53 ± 53
16	0 ± 0	47 ± 27	0 ± 0	0 ± 0	150 ± 79	0 ± 0	0 ± 0
24	0 ± 0	67 ± 15	0 ± 0	0 ± 0	30 ± 30	0 ± 0	0 ± 0
32	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	27 ± 27
64	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	13 ± 13
96	0 ± 0	157 ± 157	0 ± 0	0 ± 0	187 ± 187	47 ± 47	590 ± 34
142	0 ± 0	0 ± 0	0 ± 0	0 ± 0	73 ± 73	0 ± 0	0 ± 0
188	0 ± 0	7 ± 7	0 ± 0	0 ± 0	67 ± 67	0 ± 0	0 ± 0
365	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

isobutyric and butyric acids were detected on more than one sampling day in the absence of dimethenamid (Table 9), and acetic acid was transiently observed in the presence of dimethenamid (Table 10). The dimethenamid-free nitrate + sulfate-amended microcosms had negligible VFA formation (Table 11), but acetic acid was observed in the later sampling days in the dimethenamid-treated microcosms (Table 12). Several VFAs were observed in the autoclaved microcosms (Tables 13 and 14).

Microbial relative abundance over time and the influence of ^{14}C-herbicide treatment

RNA was also monitored in biometers that did not receive ^{14}C-dimethenamid but were prepared, sampled, and analyzed in the same manner as the biometers that were amended with ^{14}C-herbicide (Figs. 7-10).

Herbicide treatment resulted in a rapid increase in methanogen populations for both glucose-pretreated and nitrate + sulfate-amended microcosms (Fig. 7). The latter treatment sustained the population, whereas in the glucose-pretreated microcosms, methanogen levels declined to those in the herbicide untreated microcosms. As demonstrated in Fig. 2, initial methanogen populations were much higher in the glucose pretreated microcosms.

Conversely, initial concentrations of bacterial species were higher in the microcosms that were not pretreated with glucose (Fig. 8), but increased in pretreated herbicide-amended microcosms during the first 32 days of the incubation. Most of the data for bacterial populations was within the range of 70-100% for all treatments, but varied with time of incubation; therefore, neither herbicide

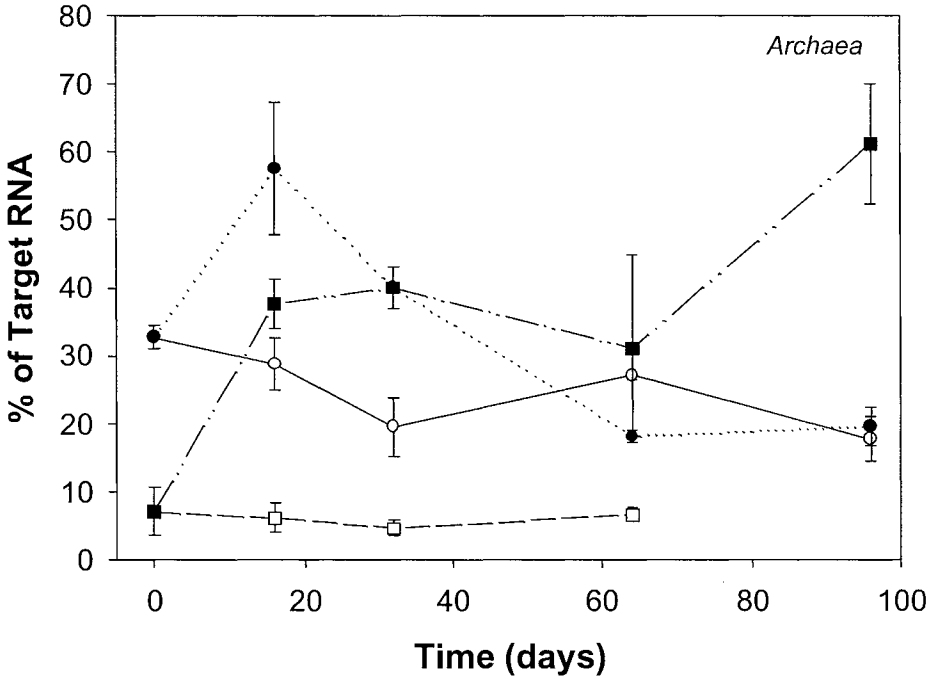

Fig. 7. Abundance of methanogens over time in soil-water microcosms. ○, pretreated for 30 d with glucose; ●, pretreated for 30 d with glucose before addition of the herbicide ^{14}C-dimethenamid; ❑, treated with nitrate + sulfate; ■, treated with nitrate + sulfate + ^{14}C-dimethenamid. Day 0 indicates the start of the experiment with herbicide addition (where appropriate) and occurs on day 30 for glucose-pretreated microcosms. The missing data for day 96 of the nitrate + sulfate treatment is due to insufficient quantities of RNA.

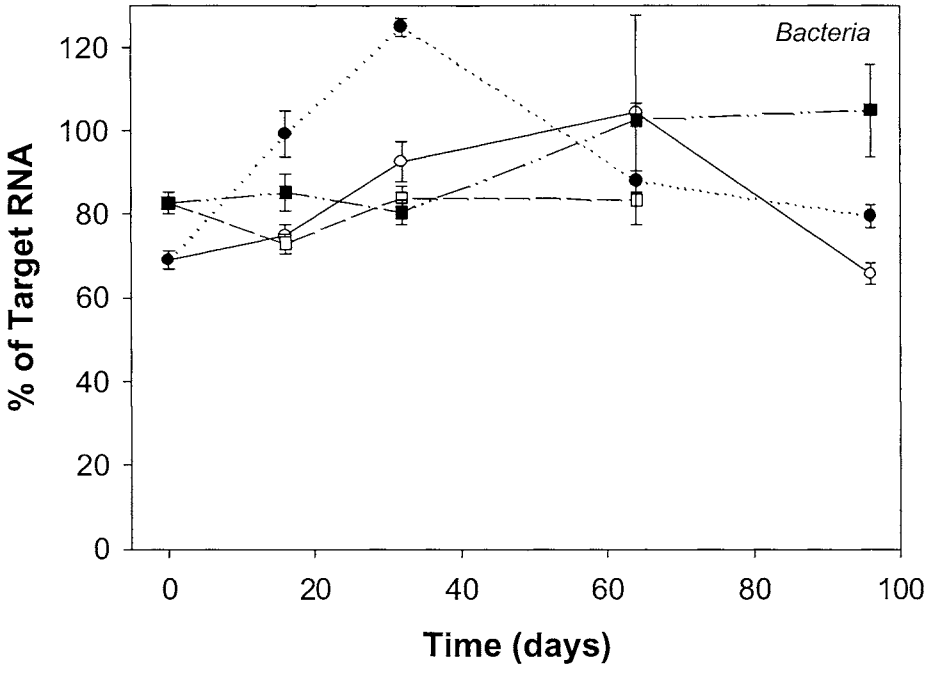

Fig. 8. Abundance of bacteria over time in soil-water microcosms. ○, pretreated for 30 d with glucose; ●, pretreated for 30 d with glucose before addition of the herbicide ^{14}C-dimethenamid; ❑, treated with nitrate + sulfate; ■, treated with nitrate + sulfate + ^{14}C-dimethenamid. Day 0 indicates the start of the experiment with herbicide addition (where appropriate) and occurs on day 30 for glucose-pretreated microcosms. The missing data for day 96 of the nitrate + sulfate treatment is due to insufficient quantities of RNA.

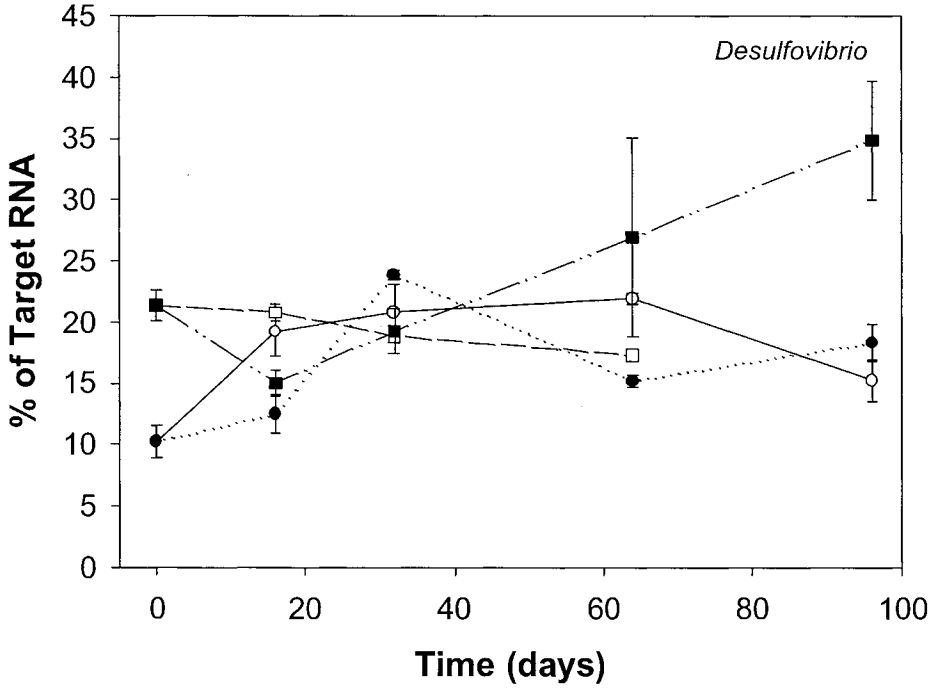

Fig. 9. Abundance of *Desulfovibrio* spp. over time in soil-water microcosms. ○, pretreated for 30 d with glucose; ●, pretreated for 30 d with glucose before addition of the herbicide ^{14}C-dimethenamid; ❑, treated with nitrate + sulfate; ■, treated with nitrate + sulfate + ^{14}C-dimethenamid. Day 0 indicates the start of the experiment with herbicide addition (where appropriate) and occurs on day 30 for glucose-pretreated microcosms. The missing data for day 96 of the nitrate + sulfate treatment is due to insufficient quantities of RNA.

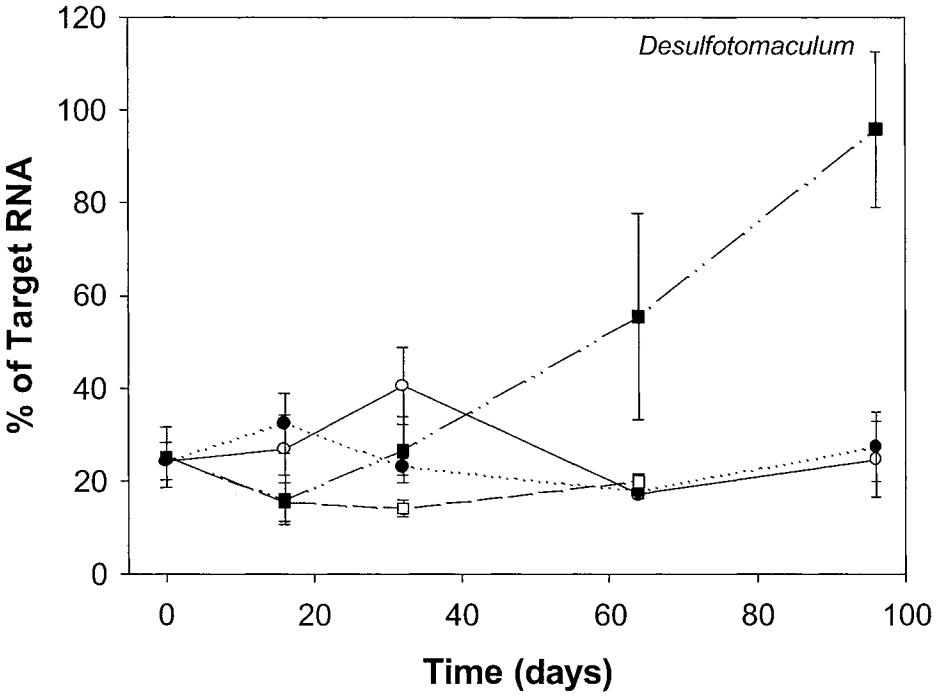

Fig. 10. Abundance of *Desulfotomaculum* spp. over time in soil-water microcosms. ○, pretreated for 30 d with glucose; ●, pretreated for 30 d with glucose before addition of the herbicide ^{14}C-dimethenamid; ❑, treated with nitrate + sulfate; ■, treated with nitrate + sulfate + ^{14}C-dimethenamid. Day 0 indicates the start of the experiment by herbicide addition (where appropriate) and occurs on day 30 for glucose-pretreated microcosms. The missing data for day 96 of the nitrate + sulfate treatment is due to insufficient quantities of RNA.

amendment nor incubation conditions appeared to adversely affect these populations. Bacterial species account for the highest number of soil organisms so these numbers are expected.

The glucose pretreatment appeared to have an adverse effect on *Desulfovibrio* spp. since levels were significantly less than in the microcosms that were not treated with glucose. After a decrease between days 0 and 16, levels of *Desulfovibrio* species increased in nitrate + sulfate-amended microcosms that were treated with ^{14}C-dimethenamid (Fig. 9).

Desulfotomaculum spp. also increased in the sulfate-containing microcosms that contained dimethenamid (Fig. 10). Similar levels of *Desulfotomaculum* were observed throughout the incubation in the other 3 treatments.

In general, increases in relative microbial abundance (Fig. 7-10) did not correspond directly with depletion of terminal electron acceptors (Tables 3-6), and may indicate the difference between measuring function and ribosomal RNA. Also, terminal electron acceptor data represents the collective activities of several populations, whereas population data, for example *Desulfovibrio* and *Desulfotomaculum*, may represent a select group of many possible genus of bacteria capable of this type of metabolism, for example sulfate reduction.

Chapter 4. Conclusions

In this study, we monitored anaerobic microbial populations and processes over time in herbicide treated and untreated soil-water microcosms. The objective was to determine the effect of incubation conditions on anaerobic populations to better understand anaerobic herbicide degradation. The use of nucleic-acid probes permitted direct monitoring of the populations. Results of the study demonstrated that methanogens and sulfate-reducing bacteria are active in anaerobic soil depending on the environmental conditions. Glucose pretreatment as a technique of creating anaerobic conditions (which is recommended by the EPA) appeared to select for methanogenic populations at the time of herbicide addition. This technique also appeared to reduce the soil population of *Desulfovibrio* spp.

Chapter 5. Recommendations

The anaerobic aquatic metabolism study is the only anaerobic fate study required by the EPA for herbicide registration. Thus it is important that this study adequately represent anaerobic environmental conditions that are exposed to herbicides, so that we understand how herbicides will behave in such environments. We recommend that the anaerobic aquatic metabolism study be modified slightly. Firstly, the experimental system should be made anaerobic by removing oxygen by conventional anaerobic techniques rather than through biological reduction since the byproducts of biological reduction (for example, consumption of oxygen with glucose utilization) appear to influence the microbial properties of the system. A procedure for using conventional anaerobic techniques to prepare and sample soil-water microcosms has been described (Crawford et al., 2000). Preserving the microbial properties of the test soil or sediment is important to linking the results of the study to real environments, thus test soil should not be air-dried before use. Moreover, a reasonable attempt should be made to characterize the dominant terminal electron accepting processes as an indication of microbial activities. Denitrification and sulfate reduction would only be expected if the test system contained unusually high levels of nitrate and sulfate which are usually limiting in soil and water that may be exposed to herbicides. Thus, soil-water test systems should be analyzed prior to study initiation to determine nitrate and sulfate concentrations, so that if these are present at elevated levels (>1 mM), they can be further monitored during herbicide degradation until they are depleted. Fe^{2+} and methane formation should be monitored throughout all anaerobic degradation studies since they occur naturally in soil without the addition of external electron acceptors. Monitoring each of these processes is relatively easy and would not require significantly more sample processing time. Correlating microbial activities (such as methane formation, Fe^{2+} formation, or sulfate reduction) to herbicide

transformation makes the study more useful by permitting extrapolation of the data to other anaerobic environments.

Following the publication of this study, we will submit the results to agrichemical companies for their input, after which we plan to submit our recommendations to the EPA with a review of relevant studies.

LITERATURE CITED

Adrian, N. R. and J. M. Suflita. 1994. Anaerobic biodegradation of halogenated and nonhalogenated *N*-, *S*-, and *O*-heterocyclic compounds in aquifer slurries. *Environ Toxicol Chem* 13:1551-1557.

Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin. 1996. The Oligonucleotide Probe Database. *Appl Environ Microbiol* 62:3557-3559.

Amann, R.I., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 59:143-169.

Amann, R. I., L. Krumholz, and D. A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol* 172:762-770.

Berry, D. F., A. J. Francis, and J.-M. Bollag. 1987. Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. *Microbiol Rev* 51:43-59.

Cozzarelli, I. M., J. S. Herman, and M. J. Baedecker. 1995. Fate of microbial metabolites of hydrocarbons in a coastal plain aquifer: the role of electron acceptors. *Environ Sci Tech* 29:458-469.

Crawford, J. J., G. K. Sims, R. L. Mulvaney, and M. Radosevich.1998. Biodegradation of atrazine under denitrifying conditions. *Appl Microbiol Biotech* 49:618-623.

Crawford, J. J., G. K. Sims, F.W. Simmons, L.M. Wax, and D.L. Freedman. Dissipation of the herbicide [^{14}C]Dimethenamid under anaerobic aquatic conditions in flooded soil microcosms. *J Agric Food Chem* (submitted).

Crawford, J. J., S. J. Traina, and O. H. Tuovinen. 2000. Bacterial degradation of atrazine in redox potential gradients in fixed-film sand columns. *Soil Sci Soc Am J* 64:624-634.

Cypionka, H. and N. Pfennig. 1986. Growth yields of *Desulfotomaculum orientis* with hydrogen in chemostat culture. *Arch Microbiol* 143:396-399.

de los Reyes, F. L, W. Ritter, and L. Raskin. 1997. Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems. *Appl Environ Microbiol* 63:1107-1117.

DeLong, E. F., G. S. Wickham, and N. R. Pace. 1989. Phylogenetic strains: ribosomal RNA-based probes for the identification of single cells. *Science* 243:1360-1363.

Freedman, D. L. and J. M. Gossett. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. *Appl Environ Microbiol* 55:2144-2151.

Genthner, B. R. S., Price, W. A., and P. H. Pritchard. 1989. Anaerobic degradation of chloroaromatic compounds in aquatic sediments under a variety of enrichment conditions. *Appl Environ Microbiol* 55:1466-1471.

Gibson, S. A. and J. M . Suflita. 1986. Extrapolation of biodegradation results to groundwater aquifer: reductive dehalogenation of aromatic compounds. *Appl Environ Microbiol* 52:681-688.

Griffin, M.E., K. D. McMahon, R. I. Mackie, and L. Raskin. 1998. Methanogenic population dynamics during startup of anaerobic digesters treating municipal solid waste and biosolids. *Biotech Bioeng* 57:342-355.

Häggblom, M. M., M. D. Rivera, and L. Y. Young. 1993. Influence of alternative electron acceptors on the anaerobic biodegradability of chlorinated phenols and benzoic acids. *Appl Environ Microbiol* 59:1162-1167.

Kaake, R. H., D. J. Roberts, T. O. Stevens, R. L. Crawford, and D. L. Crawford. 1992. Bioremediation of soils contaminated with the herbicide 2-sec-butyl-4,6-dinitrophenol (Dinoseb). *Appl Environ Microbiol* 58:1683-1689.

Loor-Vela, S. X., J. J. Crawford, F.W. Simmons, and L. Raskin. Anaerobic dissipation of [^{14}C]acetochlor in flooded soil. *Environ Sci Technol* (submitted).

Lovley, D. R. and E. J. P. Phillips. 1987. Rapid assay for microbially reducible ferric iron in aquatic sediments. *Appl Environ Microbiol* 53:1536-1540.

Mervosh, T. L., G. K. Sims, E. W. Stoller, and T. R. Ellsworth. 1995. Clomazone sorption in soil: incubation time, temperature, and soil moisture effects. *J Agric Food Chem* 43:2295-2300.

Mulvaney, R. L. 1994. Nitrogen-Inorganic Forms. In *Methods of Soil Analysis. Part 3. Chemical Methods*--SSSA Book Series No. 5. SSSA-ASA: Madison.

Ogram, A., W. Sun, F. J. Brockman, and J. K. Fredrickson. 1995. Isolation and characterization of RNA from low-biomass deep-subsurface sediments. *Appl Environ Microbiol* 61:763-768.

Raskin, L., W. C. Capman, R. Sharp, L. K. Poulsen, and D. A. Stahl. 1997. Molecular Ecology of GI Ecosystems, p. 243-298. *In* R. I. Mackie, B. A. White, and R. E. Isaacson (ed.), *Ecology and Physiology of Gastrointestinal Microbes, Volume 2*. Chapman and Hall: New York.

Raskin, L., L. K. Poulsen, D. R. Noguera, B. E. Rittmann, and D. A. Stahl. 1994. Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. *Appl Environ Microbiol* 60:1241-1248.

Raskin, L., D. Zheng, M. E. Griffin, P. G. Stroot, and P. Misra. 1995. Characterization of microbial communities in anaerobic bioreactors using molecular probes, *Antonie van Leeuwenhoek*. 68:297-308.

Rhine, E. D., G. K. Sims, R. L. Mulvaney, E. J. Pratt. 1998. Improving the Berthold reaction for determination of ammonium in soil extracts and waters. *Soil Sci Soc Am J* 62:97-102.

SAS Institute, Inc. 1988. SAS/STAT© User's Guide, Version 6.03. Cary, North Carolina.

Teske, A. C. Wawer, G. Muyzer, and N. B. Ramsing. 1996. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Appl Environ Microbiol* 62:1405-1415.

United States Environmental Protection Agency. 1982. *Pesticide Assessment Guidelines, Subpart N, Chemistry: Environmental Fate*. Environmental Protection Agency: National Technical Information Service, PB83-153973, October 18, 1982.

Vendrall, P. F. and J. Zupancic. 1990. Determination of nitrate by transnitration of salicylic acid. *Comm Soil Sci Plant Anal* 21:1705-1713.

Illinois
Department of
Natural Resources